PATENT APPLICATION

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29, 1999 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Seiichi Ishizuka, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1974 and a Ph.D. degree in 1996 from Tohoku University;

THAT I am a member of several Japanese and American Scientific Societies related to research in vitamin D and bone and mineral metabolism, a referee for two scientific journals (Biochemistry and J. Nutritional Biochemistry), and the recipient of two scientific research awards (Vitamin D research award from Brown University (1995) and scientific research award from Japanese Society for Bone and Mineral Research (1997)).

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1975, in the Department of Bone and Calcium Metabolism, where I have been involved in the study of the metabolism, biological activities and mechanism of actions of Vitamin D_3.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the Office Action of June 30, 1999 issued in reference to the application. In response to the Office Action, I have conducted the following experiments to investigate the activity of 20R-forms for the vitamin D_3 derivatives disclosed in the reference cited by the Examiner for their effect on nitroblue tetrazolium reduction activity in the HL-60 cell line compared to the instant 20S-forms for the derivatives. A description of the experiments and the results obtained are the following.

Inventive Example 1 (20S-form of vitamin D_3 derivative)

Activity for 20S-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 68, 71, 72 or 74 at

concentrations ranging from $1x10^{-9}$ M to $1x10^{-6}$ M was added to each well at 1 μl/well. Further, regarding 1 α, $25(OH)_2D_3$, an ethanol solution containing 1 x 10^{-7} M to 1 x 10^{-4} M of the compound was added at 1 μl/well, and for the control, ethanol alone was added at 1 μl/well. After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for a cytospin centrifugation. After air drying, the cell pellet was stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20S-forms of Vitamin D Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		1.5
$1\alpha,25\text{-}(OH)_2D_3$	10^{-10}	4.3±1.2
	10^{-9}	36.8±2.0
	10^{-8}	86.1±2.6
	10^{-7}	96.5±1.0
Compound (68)	10^{-12}	1.7±0.3
	10^{-11}	2.8±0.7
	10^{-10}	57.7±5.0
	10^{-9}	95.7±1.0
Compound (71)	10^{-12}	1.5±0.8
	10^{-11}	1.8±0.8
	10^{-10}	2.0±1.0
	10^{-9}	40.5±1.8
Compound (74)	10^{-12}	6.4±1.1
	10^{-11}	17.0±2.3
	10^{-10}	16.7±1.1
	19^{-9}	96.4±1.4
Compound (72)	10^{-12}	3.7±0.8
	10^{-11}	94.4±1.8
	10^{-10}	95.7±2.3
	10^{-9}	96.2±2.0

Comparative Example 1 (20R form of vitamin D_3 derivatives)

Activity for 20R-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 3, 4, 6 or 65 at concentrations ranging from $1x10^{-7}$ M to $1x10^{-4}$ M was added to each well at 1 μl/well. For the control, ethanol alone was added at 1 μl/well. After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in fresh culture medium to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for cytospin centrifugation. After air drying, the cell pellet was stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20R-forms of Vitamin D Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		5.8
Compound (65)	10^{-8} 10^{-7} 10^{-6}	6.8 65.8 88.3
Compound (3)	10^{-8} 10^{-7} $3x10^{-7}$	6.8 11.4 80.9
Compound (6)	10^{-9} 10^{-8} 10^{-7} 10^{-6}	5.2 17.0 71.2 82.5
Compound (4)	10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}	8.1 27.9 80.0 88.7 94.5

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20S-forms show excellent efficacy compared to the 20R-forms. Comparison of compound (4) with compound (72); compound (65) with compound (68); compound (6) with compound (74) and compound (3) with compound (71) reveals that the 20S-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____

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Seiichi Ishizuka